Intrathecally Administered Cholera Toxin Blocks Allodynia and Hyperalgesia in Persistent Pain Models

Robert M. Caudle, Andrew J. Mannes, Raphael Benoliel, Eli Eliav, and Michael J. Iadarola

Abstract: **In persistent pain, the spinal cord concentration of the opioid peptide dynorphin increases dramatically, yet the function of dynorphin remains unknown. If prodynorphin expression could be manipulated in vivo, it might be possible to determine what role dynorphin plays in persistent pain. Previous work in our laboratory showed that prodynorphin expression is regulated through the cyclic adenosine monophosphate pathway. Therefore, we attempted to enhance prodynorphin expression in the spinal cord of rats by stimulating adenylate cyclase with cholera toxin; however, contrary to our hypothesis, intrathecally administered cholera toxin did not enhance prodynorphin expression. Rather, cholera toxin suppressed the increase in prodynorphin produced by inflammation. Cholera toxin also inhibited the allodynia and hyperalgesia associated with inflammation and nerve injury. Interestingly, the antiallodynic and antihyperalgesic actions of cholera toxin were reversed with the opioid receptor antagonist, naloxone. These findings suggest that cholera toxin enhances or unmasks an endogenous opioid pathway to produce its antiallodynic and antihyperalgesic effects. Furthermore, these data indicate that the suppression of the inflammation-induced increase in spinal cord prodynorphin is caused by the opioid-mediated decrease in the nociceptive stimulus.**

Key words: *Chronic pain, opioids, cholera toxin, spinal cord.*

Chronic pain is a debilitating condition for millions of people that remains a major challenge for physicians. Associated with persistent pain are alterations in the expression of several gene products in the spinal cord. Some of these changes might be responsible for the most troubling symptoms of chronic pain, such as allodynia; pain to normally nonpainful stimuli; and hyperalgesia, an enhanced response to a noxious stimulus. Our laboratory has focused on analyzing the role of prodynorphin gene products in the spinal cord. During persistent nociception, the expression of the prodynorphin gene is elevated.[1-3] The enhanced expression of dynorphin in the spinal cord is highly correlated with allodynia and hyperalgesia in animal models of persistent pain; however, the subsequent role of the enhanced spinal cord dynorphin levels is currently not known. Dynorphin is an agonist at all known opioid receptors, although it has some selectivity for the κ subtype.[4] Generally, activation of opioid receptors results in the inhibition of neurotransmitter release from primary afferent terminals and hyperpolarization of dorsal horn neurons.[5-9] These actions of opioids form the basis for their analgesic effects when administered intrathecally. On the other hand, dynorphin also stimulates *N*-methyl-D-aspartate (NMDA) receptors through a nonopioid mechanism.[10-12] The enhancement of NMDA receptor function in the spinal cord by dynorphin leads to neuronal cell death and to persistent allodynia.[13-15] Because of the opioid and nonopioid actions of dynorphin, it is difficult to determine if endogenous dynorphin in the spinal cord might be contributing to or suppressing the symptoms of chronic pain. Thus, it would be desirable to regulate the expression of prodynorphin in vivo to assess its role in persistent nociception.

In the upstream regulatory sequence of the prodynorphin gene there are 4 cyclic adenosine monophosphate (cAMP) response element (CRE)-like sites, DYNCRE1, 2, 3, and 4.[16-18] In vitro studies showed that prodynorphin gene transcription is activated by the cAMP pathway through the DYNCRE3 site.[16] The sequence of the DYNCRE3 site, TGCGTCA, is similar to both the activation protein-1 (AP-1) and the CRE consensus sites, which suggests that DYNCRE3 might be regulated by both cAMP response element binding protein (CREB) and the AP-1 family of proteins. Messersmith et al[17] found in cotransfection studies that CREB repressed gene expression at the DYNCRE3 site, whereas the AP-1 binding proteins *c-jun* and *c-fos* enhanced gene transcription. Furthermore, they found that hindpaw inflammation in rats resulted in

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the phosphorylation of CREB concomitant with the enhanced expression of *c-fos*, Fos-related antigen (Fra), and phosphorylated *c-jun*. These changes in transcription factors occurred in spinal neurons that express dynorphin, and the changes preceded the increase in prodynorphin gene expression. Thus, the hypothesis was proposed that nociceptive input increases cAMP, activating protein kinase A (PKA), which leads to the phosphorylation of CREB. In turn, the CREB repression of the prodynorphin gene is relieved. The AP-1 binding proteins, which are increased during persistent nociception, then bind to DYNCRE3 to enhance prodynorphin gene transcription.[16-22] In preliminary studies, we tested the hypothesis that increasing cAMP in vivo results in an increase in prodynorphin gene expression by injecting cholera toxin intrathecally in rats. Contrary to our hypothesis, cholera toxin did not enhance prodynorphin gene expression (R.M. Caudle, A.J. Mannes, and M.J. Iadarola, unpublished observations) and, in fact, prevented the increase in spinal cord dynorphin associated with hindpaw inflammation. There are several potential reasons for these findings. The simplest explanation is that intrathecally administered cholera toxin alters nociception in some manner. Agents that reduce the nociceptive drive suppress the increase in spinal cord dynorphin associated with an injury.[19-23] Thus, the present study was designed to test the hypothesis that intrathecal cholera toxin alters nociception.

Materials and Methods

These experiments were approved by the National Institute of Dental and Craniofacial Research Animal Care and Use Committee in accordance with federal law, the regulations of the National Institutes of Health, and the guidelines of the International Association for the Study of Pain.[24]

Northern Analysis of Dynorphin Messenger RNA (mRNA)

Rats received intrathecal injections (by lumbar puncture under metafane anesthesia) of either 1 mg cholera toxin (Sigma, St Louis, MO; CAS: 9012-63-9) in 10 μL saline (n = 3) or saline (10 μL) (n = 3), followed by flushing the cannula with 10 μL saline, and an intraplantar injection of carrageenan (type 4; Sigma) (6 mg in 150 μL saline) into the left hindpaw. The animals were killed 24 hours after the injections and the lumbar enlargement was dissected in half. RNA was extracted from both the side ipsilateral to the carrageenan injection as well as the side of the spinal cord contralateral to the injection. RNA was loaded (15 μg per well) and probed for dynorphin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as previously described.[2] GAPDH was used to normalize lane loading. All blots were run in triplicate, one cholera toxin– and one saline-injected rat per blot, and quantified by using a phosphoimager. The densitometry data from each blot were normalized to the half of the spinal cord obtained from the noninjured side of the saline-injected rats.

Carrageenan-Induced Inflammation

Male Sprague-Dawley rats weighing 250 to 350 g were caged in pairs and allowed food and water ad libitum. The rats were anesthetized with a combination of xylazine (10 mg/kg) and ketamine (50 mg/kg) by intraperitoneal (IP) injection. Then, using sterile technique, an intrathecal (IT) catheter (PE-10 Intramedic Polyethylene tubing, Becton Dickinson, Franklin Lakes, NJ) was placed into the IT space as previously described.[25] Any animals showing motor deficits after the placement of the cannula were euthanized and not included in the study.

Time course experiments were performed by administering 1 mg cholera toxin (IT, n = 6) in 10 μL saline or saline alone (IT, n = 6) at the same time as the induction of paw inflammation with 6 mg carrageenan in 150 μL sterile saline solution. The carrageenan injection was into the midplantar region of the left hindpaw. These animals were tested before the injections and at 8, 24, and 48 hours after the injections.

Dose-response experiments were performed by using IT cholera toxin (0 to 6 mg) (n = 6 per dose, total = 24 rats) injected 24 hours before hindpaw inflammation with carrageenan. The animals were tested 4 hours after the carrageenan injection.

To compare the actions of cholera toxin on multiple nociceptive tests, cholera toxin (1 mg in 10 μL saline) (n = 10), the β-fragment of cholera toxin (Sigma) (1 mg in 10 μL saline) (n = 10), or saline (10 mL) (n = 10) was injected into the IT space through the cannula 24 hours after the implantation. The β-fragment of cholera toxin was included as a control for binding of the whole toxin to the cell membranes. The cannulas were then flushed with another 10 μL of saline. Twenty-four hours after the IT injection, the left hindpaws of the rats were inflamed by using carrageenan. The animals underwent nociceptive testing before the carrageenan injection, 1 and 4 hours after the injection. Naloxone (Sigma) (1 mg/kg, IP) was administered immediately after the last nociceptive test at the 4-hour time point. The rats were then tested 30 minutes after the naloxone injection. Cholera toxin, the β-fragment, and the saline injected groups were run in parallel. All animals were returned to their cages and allowed food and water between testing.

Chronic Constriction Injury

Rats were anesthetized with sodium pentobarbital (50 mg/kg, IP) and the left sciatic nerve was exposed in the midthigh area. Four loose ligatures of chromic cat gut suture were placed around the nerve, as described by Bennett and Xie.[26] The wound was then closed in layers. Before the surgery and 9 days after the surgery, the animals were tested by using the nociceptive assays. After the test on day 9, the animals were injected by lumbar puncture under metafane

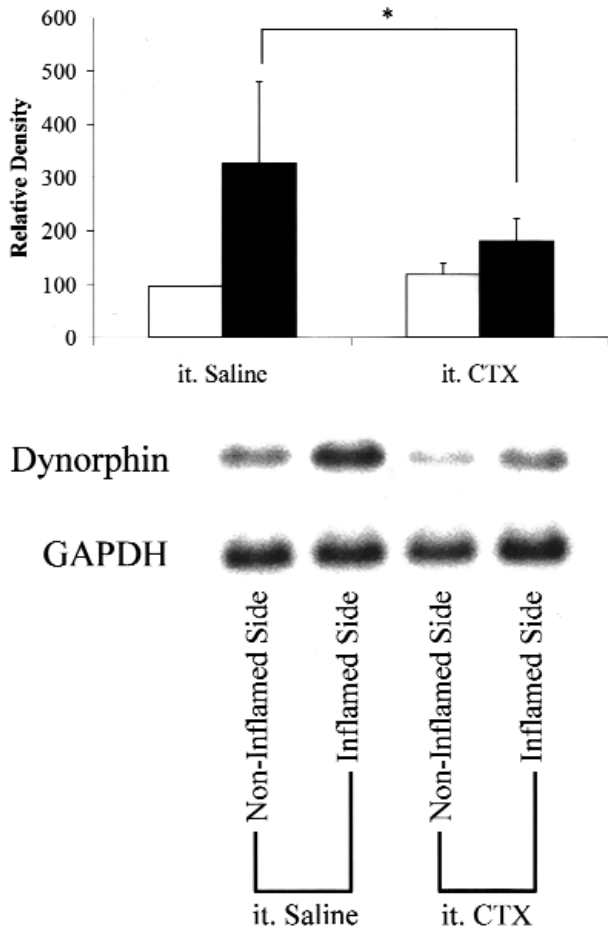

Figure 1. Intrathecal cholera toxin (CTX) attenuates carrageenan-induced increases in spinal cord dynorphin mRNA expression. Northern analysis was performed on tissue from the inflamed (■) and noninflamed (□) sides of the rats' lumbar spinal cords as described in the methods section. The tissue was collected from rats that received either IT cholera toxin (n = 3 rats) or IT saline (n = 3 rats). The mRNA was quantified using a phosphoimager. Lane loading was monitored by using GAPDH as the internal standard. Data were normalized to the hybridization observed in the lane from the noninflamed side of the saline-treated rats' spinal cords. The bars represent means ± SEM. Asterisk indicates $P < .05$ (*t*-test, cholera toxin–treated *v* saline-treated animals).

anesthesia with either cholera toxin (1 mg in 10 μL saline) (n = 10) or with saline (10 μL) (n = 10). The animals were tested again 24 hours after the IT injection. They then received IP injections of naloxone (1 mg/kg) and were again tested 30 minutes after the naloxone injection.

Nociceptive Tests

The animals were subjected to 3 nociceptive measures for heat hyperalgesia, cold allodynia, and mechanical hyperalgesia. The order of the cold allodynia and mechanical hyperalgesia assays were randomized; however, the heat hyperalgesia assay was always performed last. Because the area heated by the light source covered a substantial portion of the paw's plantar surface, it was possible that the heat could potentiate hypersensitivity in the other assays. A minimum of 10 minutes separated the testing procedures to further reduce the influence of prior nociceptive testing. The investigator performing the nociceptive tests was blinded to the treatment each rat received.

Heat Hyperalgesia

Heat hyperalgesia was assayed by paw withdrawal latency from a radiant heat source, as described previously.[27] Data were expressed as raw withdrawal latencies for the paws ipsilateral and contralateral to the injury.

Cold Allodynia

Cold allodynia was tested by an acetone spray test that was modified from that described previously.[28] Rats were placed on a grating and 250 μL of acetone was squirted onto the midplantar skin of the hindpaws. The duration of the withdrawal evoked by the evaporative cooling was timed with a stopwatch.

Mechanohyperalgesia

Mechanohyperalgesia was assayed with a pinprick test as previously described.[29] A rat was placed on an elevated grating, and the tip of a safety pin was pushed slowly against the midplantar surface of the hindpaw until the skin was dimpled but not penetrated. The duration of the pinprick-evoked nociceptive withdrawal was timed with a stopwatch. Normal responses were usually very rapid and were too quick to time accurately by hand. Therefore, we arbitrarily assigned normal responses a duration of 0.5 seconds.

Statistics

All data are expressed as means ± standard error of mean (SEM). One- and 2-way analyses of variance (ANOVAs) were used when appropriate, and Bonferroni's post hoc tests were used for individual comparisons. When comparisons were made between 2 distinct means, *t*-tests were performed. Significance was assigned to a *P* value of less than or equal to .05. The statistical software package PRISM (Graphpad Software Inc, San Diego, CA) was used for the analyses.

Results

Northern Analysis of Dynorphin mRNA

Preliminary results with IT-injected cholera toxin in naive rats failed to produce a change in prodynorphin gene expression (R.M. Caudle, A.J. Mannes, and M.J. Iadarola, unpublished observations). Therefore, experiments were performed in rats with a unilateral hindpaw inflammation, which was previously shown to stimulate the transcription and translation of the prodynorphin gene.[1,2,17] Twenty-four hours after the IT injection of cholera toxin and saline and the intraplantar injection of carrageenan, RNA was harvested from the lumbar spinal

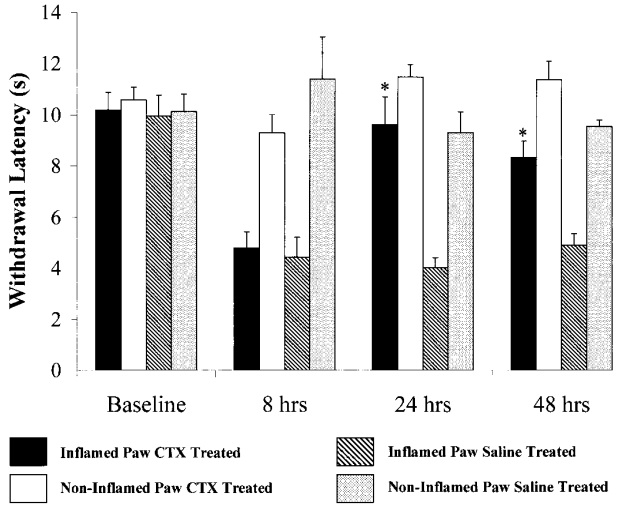

Figure 2. Time course for IT-administered cholera toxin on carrageenan-induced heat hyperalgesia. IT Cholera toxin (1 mg) (n = 6 rats) or IT saline (n = 6 rats) was administered at the same time that carrageenan was injected into the left hindpaw. Heat hyperalgesia was then measured at the times indicated. The bars represent means + SEM. Asterisk indicates $P < .05$ (ANOVA followed by Bonferroni's test, cholera toxin [CTX]–treated *v* saline-treated).

cords of the animals, taking care to separate the tissue from the sides ipsilateral and contralateral to the hindpaw inflammation. Northern blots were performed by using probes to prodynorphin and GAPDH. The Northern blot in Figure 1 shows that IT-administered cholera toxin suppressed the ipsilateral increase in prodynorphin mRNA that accompanies hindpaw inflammation. The Northern blots were quantified by using densitometry and plotted as the graph in Figure 1. Cholera toxin significantly suppressed the inflammation-induced increase in prodynorphin mRNA on the side of the lumbar spinal cord that was ipsilateral to the inflammation ($P < .05$, *t*-test, inflamed paw cholera toxin–treated *v* inflamed paw saline-treated). Cholera toxin did not increase the expression of prodynorphin on the contralateral side of the spinal cord; thus, contrary to our original hypothesis, cholera toxin did not induce transcription of the prodynorphin gene in vivo but, in fact, suppressed an increase in expression. This finding led to the main hypothesis of this study—namely, IT cholera toxin alters nociceptive processing.

Cholera Toxin and Inflammation

Carrageenan-induced hindpaw inflammation produces a decrease in the withdrawal latency to heat (Fig 2). Cholera toxin (1 mg) administered at the same time as the carrageenan did not alter the responses to the nociceptive tests until the 24-hour time point ($P < .05$, ANOVA, cholera toxin–treated *v* saline-treated). Thus, for the remainder of the experiments, the cholera toxin was administered 24 hours before measuring its effects.

The dose-response relationship for cholera toxin (Fig 3) showed that 1 mg of cholera toxin administered IT 24 hours before the induction of inflammation produced a maximum effect on the heat hyperalgesia test. Dose response relationships were not performed with the other assays. Because 1 mg cholera toxin produced a maximum effect, this dose was used as our standard.

Cholera toxin administered IT 24 hours before the carrageenan injection suppressed the decrease in paw withdrawal latency to heat (Fig 4A) ($P < .05$, ANOVA, cholera toxin–treated *v* saline-treated). In addition, cholera toxin prevented the increase in withdrawal duration after the cooling of the inflamed paw with acetone (Fig 4B) ($P < .05$, ANOVA, cholera toxin–treated *v* saline-treated) or probing with a safety pin (Fig 4C) ($P < .05$, ANOVA, cholera toxin–treated *v* saline-treated). Interestingly, the pretreatment with cholera toxin did not influence baseline responses in these nociceptive assays before the carrageenan injection.

Cholera toxin is composed of 2 regions known as the binding portion, or β-fragment, and the catalytic fragment. The catalytic fragment is responsible for adenosine diphosphate (ADP)–ribosylating G_s. The β-fragment binds to monosialotetrahexylganglioside and is known to induce arachidonic acid metabolism and calcium influx through cAMP-independent mechanisms.[30-32] To determine if the catalytic portion of cholera toxin was necessary for its actions, the binding portion was administered alone. Injection of the β-fragment of cholera toxin 24 hours before the induction of inflammation had no effect on inflammation-induced allodynia and hyperalgesia on any of the tests. Figure 4D shows the lack of effect of the β-fragment in the mechanical hyperalgesia assay, which was the assay most sensitive to the effects of cholera toxin.

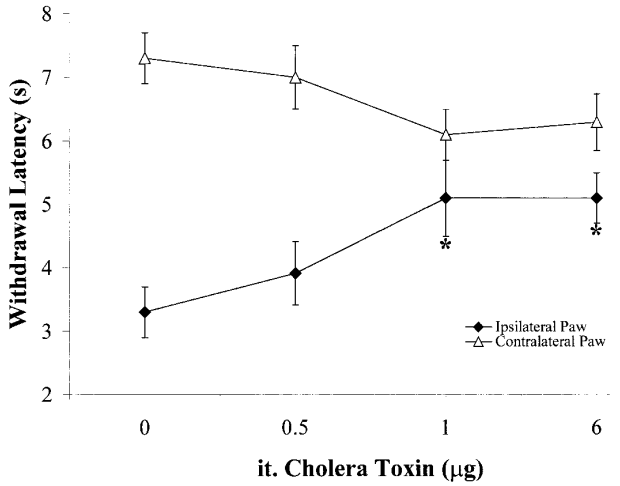

Figure 3. Dose-response relationship for IT cholera toxin on carrageenan-induced heat hyperalgesia. Rats received IT injections of various doses of cholera toxin (n = 6 rats per dose, 24 rats total) in saline 24 hours before carrageenan injection into the left hindpaw. Data were collected as stated in the methods section and presented as raw withdrawal latencies. The data presented in the graph are from the 4-hour time point after the carrageenan injection. The data points represent means + SEM. * $P < .05$ (ANOVA followed by Bonferroni's test when compared with saline-treated animals).

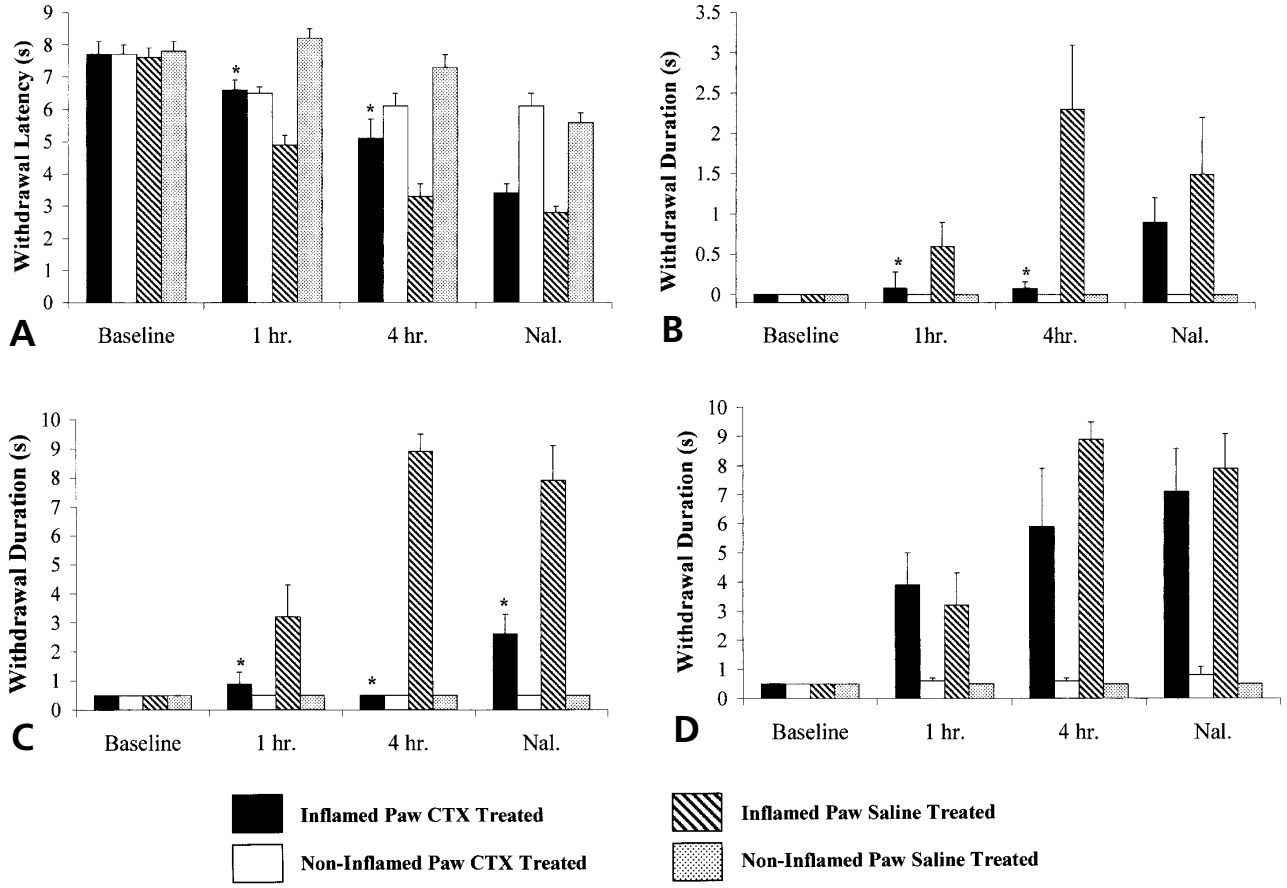

Figure 4. Intrathecal cholera toxin prevents inflammation-induced hyperalgesia and allodynia. Cholera toxin (CTX) (1 mg) (n = 10 rats), saline (n = 10 rats), or the β-fragment of cholera toxin (n = 10 rats) was IT-administered 24 hours before paw inflammation with carrageenan and nociceptive testing. Nociceptive measures were performed before, 1 hour, and 4 hours after carrageenan injection. After the testing at 4 hours, the animals received intraperitoneal (IP) injections of naloxone (Nal.) (1 mg/kg). The animals were tested again 30 minutes after the naloxone injection. The bars represent means + SEM. Asterisk indicates $P < .05$ (ANOVA followed by Bonferroni's test, cholera toxin–treated *v* saline-treated animals). (A) Heat hyperalgesia was measured by determining the latency for paw withdrawal from a radiant heat source. (B) Cold allodynia was determined by measuring paw withdrawal duration stimulated by acetone-induced evaporative cooling of the paw. (C) Mechanical hyperalgesia was determined by measuring paw withdrawal duration following a pinprick. (D) The β-fragment of cholera toxin did not alter mechanical hyperalgesia.

After the nociceptive measurements at the 4-hour time point, the animals were given an IP injection of the opioid receptor antagonist, naloxone (1 mg/kg). The animals were then retested 30 minutes later. As shown in Figure 4, naloxone reversed the antihyperalgesic and antiallodynic effects of IT cholera toxin but had no effect on saline- or β-fragment–pretreated animals. These findings indicate that the antihyperalgesic and antiallodynic actions of cholera toxin were mediated through opioid receptors.

Cholera Toxin and Nerve Injury

In the inflammation model, the cholera toxin was administered preemptively to prevent the hyperalgesia and allodynia. The next question addressed was whether cholera toxin could reverse these symptoms in an established pain syndrome. The chronic nerve constriction injury (CCI) model was chosen for these experiments. The CCI produced a decrease in withdrawal latency to heat in the paw ipsilateral to the nerve injury (Fig 5A). Likewise, the injury produced an increase in paw withdrawal duration in response to acetone cooling or pinprick in the paw ipsilateral to the injury (Figs 5B, 5C). Twenty-four hours after the IT injection of cholera toxin, the hyperalgesia and allodynia associated with the CCI were attenuated in all 3 nociceptive tests when compared with either saline-treated animals or to the withdrawal latencies or durations obtained before cholera toxin treatment. IT cholera toxin did not influence the nociceptive responses of the contralateral paws. Again, naloxone (1 mg/kg, IP) reversed the antiallodynic and antihyperalgesic actions of cholera toxin, as it did in the inflammation model, indicating the involvement of opioid receptors.

Discussion

The initial hypothesis in this study was that elevation of cAMP in the spinal cord would produce an increase in the expression of prodynorphin gene products. This hypothesis stems from studies showing that the DYNCRE3 element in the prodynorphin promoter can bind both

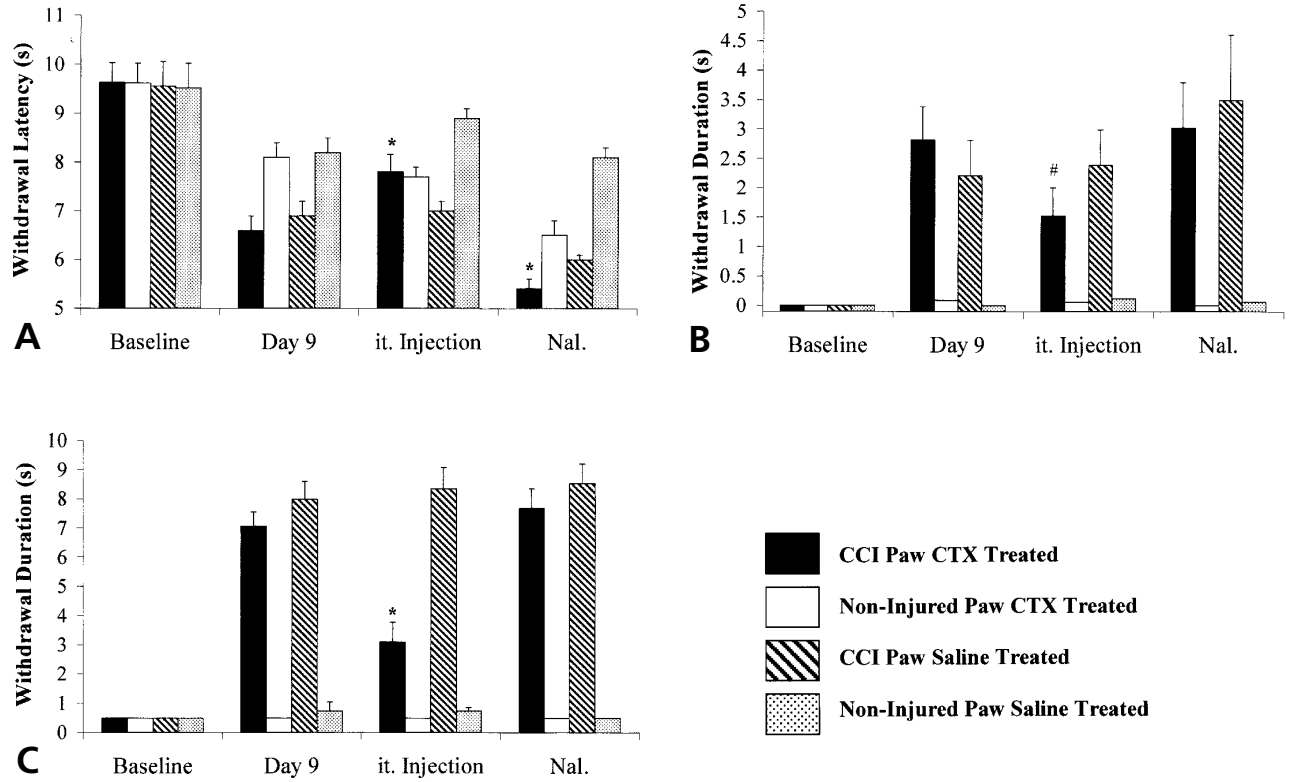

Figure 5. IT cholera toxin reverses CCI-induced hyperalgesia and allodynia. Nociceptive measures were performed before surgery and on days 9 and 10 after CCI surgery. IT cholera toxin (1 mg) (n = 10 rats) or IT saline (n = 10 rats) was administered immediately after the testing on day 9, and the animals were then retested on day 10. Naloxone (Nal.) (1 mg/kg, IP) was administered after the testing on day 10. The animals were retested 30 minutes after the naloxone injection. The bars represent means ± SEM. (A) Heat hyperalgesia (* $P < .05$ when compared with the injured paw's latency on day 9, ANOVA followed by Bonferroni's test). (B) Cold allodynia ($^{\#}$ $P < .05$ paired *t*-test day 9 treated *v* IT injection for the cholera toxin–treated animals). (C) Mechanical hyperalgesia was measured as described in the methods section and in Figure 4. * $P < .05$ (ANOVA followed by Bonferroni's test, cholera toxin [CTX]–treated *v* saline-treated animals).

CREB and AP-1 binding proteins.[16-18] CREB binding represses the expression of the gene, whereas binding of *c-fos* and *c-jun* enhances gene expression. In transient transfection experiments, stimulation of adenylate cyclase with forskolin resulted in enhanced gene expression through the DYNCRE3 site.[17] In the present study, cholera toxin was used because it ADP-ribosylates G_s, resulting in the persistent activation of adenylate cyclase.[33] Presumably, the elevation of cAMP would enhance the activity of PKA, resulting in the phosphorylation of CREB (P-CREB) and relief of CREB repression of the prodynorphin gene.[17] Membrane-permeable cAMP analogs were considered for these experiments, but these agents would diffuse from the site of administration fairly quickly. To influence prodynorphin transcription, cAMP levels would have to remain elevated for some time.

Preliminary experiments in naive animals showed that IT-administered cholera toxin had no effect on prodynorphin gene expression (R.M. Caudle, A.J. Mannes, and M.J. Iadarola, unpublished observations). This result was not totally unexpected. CREB represses the expression, whereas AP-1 binding proteins stimulate expression of prodynorphin.[17, 18] Activation of PKA with cholera toxin would phosphorylate CREB, relieving the CREB block at the DYNCRE3 site. However, PKA would not necessarily stimulate the expression of AP-1 binding proteins such as *c-fos* in vivo. Thus, the cholera toxin might provide no positive signal for prodynorphin expression. In a previous study, it was found that hindpaw inflammation produced a bilateral increase in P-CREB, whereas *c-fos* is elevated only ipsilateral to the inflammation.[17] P-CREB is believed to be required for expression of the *c-fos* gene through a CRE element at –60 in the promoter.[34] However, P-CREB is not sufficient in itself for initiating *c-fos* transcription; some other unknown factor is required that is provided by the nociceptive afferent input.[17] Thus, we used carrageenan hindpaw inflammation in the rat to provide a nociceptive stimulus. In these experiments, we found, much to our surprise, that IT cholera toxin did not enhance the levels of prodynorphin mRNA in the lumbar spinal cord. In fact, the Northern analysis revealed that cholera toxin suppressed the increase in prodynorphin mRNA usually associated with hindpaw inflammation (Fig 1). The cholera toxin did not increase prodynorphin mRNA on the side of the spinal cord contralateral to the inflammation, which is consistent with our observations in naive animals. This finding disproved our original hypothesis and led to the main hypothesis of this study,

which was that IT-administered cholera toxin alters nociceptive processing.

It is well documented that IT-administered analgesics can block nociception-induced increases in spinal cord *c-fos* and dynorphin.[19-23] Therefore, it was possible that IT-administered cholera toxin had an analgesic action that suppressed transmission of nociceptive information, thus, preventing the expression of AP-1 binding proteins and prodynorphin. Figure 4 shows that IT cholera toxin blocked allodynia and hyperalgesia in the rat hindpaw inflammation model, and Figure 5 shows that cholera toxin reverses these symptoms in the CCI model. These findings suggest that the reason for cholera toxin not enhancing prodynorphin expression is the suppression of the nociceptive signal or, more likely, an inhibition of excitatory processes that led to allodynia and hyperalgesia. This finding is most remarkable because the IT injection of membrane-permeable cAMP analogs produces hyperalgesia and allodynia, whereas inhibitors of adenylate cyclase and PKA suppress capsaicin-induced hyperalgesia and allodynia.[35] Our data contradict the cAMP analog study, suggesting that by using cholera toxin we inadvertently activated another mechanism that suppresses the cAMP-induced allodynia and hyperalgesia. This conclusion is supported by our finding that the antiallodynic and antihyperalgesic actions of cholera toxin were reversed by the opioid antagonist naloxone. Our finding that IT cholera toxin has antiallodynic and antihyperalgesic properties was confirmed by a recent communication by Chung et al that showed that IT cholera toxin could block excitatory amino acid–induced allodynia and hyperalgesia in mice.[36]

The dose-response relationship for cholera toxin (Fig 3) shows that 1 mg is maximally effective, whereas 0.5 mg is either noneffective or just below detectable levels. This is a remarkably steep dose-response relationship, suggesting that a threshold concentration must be reached to produce an effect that is maximal. Below that threshold, no effect is observed. This result is consistent with the enzymatic function of cholera toxin because a few molecules of the enzyme within a cell might be all that are needed to ADP-ribosylate all the G_s. The inability of the β-fragment of cholera toxin to mimic the actions of the whole toxin also indicates that ADP-ribosylation of G_s is the most likely mechanism for cholera toxin's antiallodynic and antihyperalgesic effects.

Another interesting observation that can be seen in the dose-response relationship and in the heat hyperalgesia assays for both the inflammation (Fig 4A) and CCI (Fig 5A) models is that as cholera toxin increases the latency to withdrawal on the injured paw, the latency to withdrawal on the contralateral side decreases. This effect did not reach statistical significance in any of the assays; however, the trend in the 3 experiments suggests that as the hyperalgesia and allodynia are relieved by cholera toxin, the animals are less reluctant to transfer weight from the contralateral paw to the injured paw during testing. This result is important because many investigators, ourselves included, have published their results as difference scores. These scores are obtained by subtracting the withdrawal latencies of the 2 hindpaws. This form of data manipulation tends to exaggerate the response of the animal to the treatment by assigning the behavioral response observed in the noninjured limb to the injured limb.

There are 2 mechanisms that we speculate might lead to the effects observed with IT-administered cholera toxin. One explanation for cholera toxin activating, or unmasking, an endogenous opioid system is that the cholera toxin–activated G_s enhances the release of endogenous opioid peptides. G_s was previously shown to couple directly to voltage-gated calcium channels to enhance current flow through the ionophore.[37] If this occurred on presynaptic terminals in the spinal cords of our animals, it is possible that the release of opioid peptides was enhanced. This idea remains to be tested.

On the postsynaptic side, G-protein–coupled receptors, such as opioid receptors, are internalized and desensitized by their endogenous peptide agonists.[38-40] If endogenous opioid peptides released by persistent pain[41-46] induce opioid receptor internalization and desensitization, as was shown for substance P and NK-1 receptors,[47,48] then it is possible that cholera toxin blocks or reverses the desensitization. The mitogen-activated protein kinase (MAPK), or extracellular signal-regulated kinase (ERK), pathway was reported to be required for opioid receptor internalization and desensitization.[49] Increased levels of cAMP inhibit the ERK pathway.[50] Thus, cholera toxin might have prevented opioid receptor desensitization by inhibiting ERK-mediated internalization. Ji et al recently showed that blocking the ERK pathway with selective inhibitors blocks formalin-induced allodynia and hyperalgesia.[51] These data are consistent with our cholera toxin data and the opioid receptor internalization hypothesis. Unfortunately, Ji et al did not use an opioid receptor antagonist in their study to determine if opioids were involved.

The concept that elevated cAMP would block opioid receptor internalization and desensitization seems at odds with current opiate tolerance theory in which elevations in cAMP are associated with reduced, rather than enhanced, opiate efficacy.[52] However, receptor internalization and desensitization to endogenous opioid peptides are distinctly different processes from morphine-induced tolerance (see Whistler et al for discussion[40]). A major difference between the processes is that morphine does not stimulate receptor internalization but is highly effective at inducing tolerance. Endogenous peptides, on the other hand, are very effective at receptor internalization but do not induce tolerance as readily as morphine.[40] Also, the elevation in cAMP produced by tolerance to morphine requires the de novo synthesis of adenylate cyclase.[52] Several hours of exposure to opioids would be required before cAMP levels were elevated by opioids. We found that cholera toxin's effects could be observed within 1 hour of the initiation of hindpaw inflammation (Fig 4). This length of time is insufficient for significant synthesis of

adenylate cyclase; thus, tolerance to endogenous opioid peptides was not likely to be a significant factor in cholera toxin's actions. Receptor internalization and desensitization require only a few minutes of peptide exposure.[47, 48] Therefore, if cholera toxin influences the function of opioid receptors, it is likely disrupting the internalization and desensitization process.

The opioid peptide and opioid receptor mediating IT cholera toxin's antiallodynic and antihyperalgesic actions are not known. Acute nociception studies have shown the release of enkephalin, dynorphin, and endomorphin in the spinal cord.[41-46] Yet, in persistent nociception studies, dynorphin release remained elevated, whereas enkephalin release was suppressed.[3] The release of endomorphins in persistent nociception has not been studied. In addition, spinal cord μ and δ opioid receptors have been shown to be effective targets for managing persistent pain, whereas κ_1 opioid receptors are not particularly useful in the inflammation or CCI models.[13,25,53] These data suggest that endogenously released dynorphin or endomorphin might be acting on μ or δ opioid receptors to produce cholera toxin's effects in the persistent pain models. Alternatively, we have found that an opioid receptor, which we label the κ_2 opioid receptor based on pharmacology and receptor binding, is highly effective at blocking hyperalgesia and allodynia but does not produce analgesia.[11,25,28,54] The κ_2 receptor is highly abundant in the spinal cords of rodents and primates including humans.[54] It is possible that the endogenous peptides are acting through the κ_2 receptor to mediate cholera toxin's actions on hyperalgesia and allodynia. However, because naloxone is a nonselective opioid antagonist, our data do not shed any particular light on which of the peptides and receptors are involved in cholera toxin's actions.

A comparison of the effects of cholera toxin on the 2 pain models points to some interesting observations. Cholera toxin was not quite as effective at reversing allodynia and hyperalgesia in the CCI model as it was at preventing these symptoms in the inflammation model. This is interesting because the changes in paw withdrawal latency and duration in the 3 assays are not as large in the CCI model as they are in the inflammation model. This is particularly true of the heat hyperalgesia assay. As a less robust pain model, it would seem that cholera toxin would be more effective in CCI; however, allodynia and hyperalgesia are somewhat resistant to opioid treatment in the CCI model,[55, 56] and our data suggest that cholera toxin inhibits allodynia and hyperalgesia through an opioid mechanism. Thus, it is possible that the CCI model's resistance to opioids makes the model less sensitive to cholera toxin because cholera toxin's effects are mediated by endogenous opioids.

Finally, cholera toxin activates G_s to stimulate the production of cAMP.[33] This pathway influences the activity of many intracellular processes; therefore, it is not likely that the effects of IT-administered cholera toxins are limited to the opioid system. Other neuropeptide and neurotransmitter systems, such as substance P and serotonin, probably are influenced also by cholera toxin. However, the opioid antagonist naloxone reverses the antiallodynic and antihyperalgesic effects of IT cholera toxin. This finding suggests that our behavioral assay is particularly sensitive to the opioid system, but we have not yet used antagonists to other neurotransmitters. We might find that other neurotransmitters are involved in cholera toxin's antiallodynic and antihyperalgesic effects as well as the opioids. The effect of cholera toxin on nonopioid neurotransmitter pathways in the spinal cord is an interesting topic that remains to be studied.

In summary, our data show that the IT injection of cholera toxin in rat models of persistent pain blocks or reverses hyperalgesia and allodynia. These effects of cholera toxin are antagonized by naloxone, indicating that the endogenous opioid system is involved. The mechanism by which cholera toxin produces its antihyperalgesic and antiallodynic effects remains an active area of investigation.

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